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Constitutive expression of the T cell costimulatory molecules B7-1 and B7-2 on tumor cells induced antitumor immunity in a variety of tumor models. By contrast, expression of B7-1 or B7-2 on 66.1 and 410.4 mammary carcinoma cell lines resulted in significant delay in their growth, but not complete rejection. However, multiple injections of growth arrested B7-1 transfectants induced protective immunity in about 50% of mice. Administration of small amounts of recombinant IL-12 further enhanced the immunogenicity of B7-1 transfectants leading to their rejection, and more importantly prevented metastasis following a subsequent challenge with fairly high dose of wild type tumor cells. These observations indicate that a combination of B7-1 and IL-12 was able to mobilize the effector cells resulting in potent antitumor immunity. Further studies are under way to investigate the effect of expression of MHC class II molecules either alone or together with B7-1 or B7-2 on the induction of antitumor immunity. This will help understand the role of CD4+ T cells in the development and maintenance of antitumor immunity.

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(5) INTRODUCTION:

The identification and characterization of many tumor antigens and the parallel explosion of knowledge in understanding the cellular and molecular mechanisms of antigen recognition by the immune system have given renewed hopes to envisage immunotherapy as a promising modality to treat certain tumors (1-5). Initial trials of immunotherapy of cancer in general, breast cancer in particular, used rather non-specific immunostimulators ranging from Bacillus Calmette-Guerin (BCG) and levamisole to interferon, interleukins and monoclonal antibodies (mAb) (6-8). The purpose of the present study is to genetically modify the tumor cells so as to facilitate their recognition by the host's tumor-specific T lymphocytes and induce efficient and vigorous anti-tumor immunity. Such gene-modified tumor cells can be used as cellular vaccine to treat human cancers.

As known with nominal antigens, it is reasonable to speculate that anti-tumor immune response will involve recognition of the "tumor antigen" by $CD4^+$ helper T cells (Th) and $CD8^+$ cytotoxic T cells (Tc or CTL) in the context of MHC class II and class I molecules, respectively (3-5). Clonal expansion of these T cells and their subsequent functional maturation is governed by cytokines and other accessory molecules present on APC (9). Some of these cytokines besides being autocrine growth factors, also enhance the cytotoxicity potency of the effector cells (10, 11).

Cytokines and tumor immunity:

A variety of cytokines including IL-2, IL-4, IL-10, IL-12, TNFa, IFNg and GM-CSF have been used to augment tumor regression *in vivo* (12). In some cases the therapeutically effective concentrations of cytokines are accompanied by toxic side effects. This problem was overcome by cytokine gene therapy, in which tumor cells were transfected with cytokine genes and a sufficient amount of cytokine was released at the tumor site with out leading to high systemic levels (13-16). However, the anti-tumor immunity induced was relatively short-lived and the efficacy varied depending on the tumor model under study (17).

Tumor immunity by expression of MHC class II:

While $CD8^+$ T cells are the major effector cells in killing the target tumor cells, their proliferation and functional maturation requires help from $CD4^+$ T cells. Since MHC class II gene expression is tightly regulated and restricted to

professional APCs, the majority of tumor cells do not express MHC class II proteins and can not stimulate $CD4^+$ T cells. As a consequence, in the absence of $CD4^+$ T cell help, the $CD8^+$ T cells are not sufficiently matured or activated. We and others have shown that constitutive expression of MHC class II genes in tumor cells resulted in rejection of the tumor cells by a syngenic host (18-21). Rejection of class II^+ tranfectants resulted in the induction of protective immunity against wild type tumor cells (18-21). These findings strongly suggest that constitutive expression of MHC class II molecules on tumor cells enable them to directly present the tumor peptides to $CD4^+$ tumor-specific Th cells leading to potent anti-tumor immunity (22).

Costimulation of T cells and tumor immunity:

Although the engagement of T cell antigen receptor (TCR) with the antigen/MHC complex is a necessary primary signal for T cell activation, maximal activation of T cells requires additional costimulatory signal(s) (23-25). In fact, in the absence of a costimulatory signal T cells have been shown to enter a state of antigen-specific anergy or deletion (26-28). A variety of cell surface molecules have been shown to deliver costimulatory signals. One of the well studied costimulatory molecules is the B7 family of proteins. Two independently regulated gene products have been identified as members of the B7 family, B7-1 (CD80) and B7-2 (CD86) and these proteins do not share any significant sequence homology (29-31). Both B7-1 and B7-2 have been shown to bind two different receptors on T cells namely CD28 and CTLA-4 (32, 33). Upon receiving the antigen-specific and costimulatory signals both $CD4^+$ and $CD8^+$ T cells undergo further differentiation, enhanced proliferation and secrete elevated levels of cytokines (34-36). We and others have shown that constitutive expression of B7-1 and B7-2 molecules by tumor cells resulted in the rejection of these gene-modified tumor cells and prior injection of $B7^+$ tumors induced protective immunity against subsequent wild type tumor challenges in syngenic hosts (37-39). In addition, a synergistic effect was observed when tumor cells were gene modified to express both MHC class II and B7-1 molecules; thee cells are a potent vaccine capable of inducing complete regression of previously established tumors (40).

Based on these reports, the goal of the present study is to investigate the effect of constitutive expression of MHC class II and costimulatory molecules,

B7-1 and B7-2 in enhancing anti-tumor immunity against murine mammary carcinoma as a model for human breast cancer.

Tumor Model:

Murine mammary carcinoma cell lines, 66.1 (metastatic and non-immunogenic) and 410.4 (non-metastatic and weakly immunogenic) were used in the current studies. These tumor cell lines were derived from a parental spontaneous tumor in Balb/c mice (40, 41). They both express normal levels of MHC class I molecules but do not express MHC class II molecules or the costimulatory molecules B7-1 and B7-2. These criteria bring these two tumor cell lines closer to human breast cancer and serve as a model system to study.

The wild type tumor cell lines 410.4/WT and 66.1/WT were transfected with plasmid vectors containing cDNA encoding murine B7-1, B7-2 proteins and/or cDNA encoding syngenic MHC class II alpha and beta polypeptides forming Iad molecules. Cotransfection with plasmid containing a neomycin resistance gene was performed in order to allow selection of transfectants from the wild type tumor cells. Transfections were performed using Lipofectine or Lipofectamine as previously described (18, 39). Transfectants were grown in medium containing the selection drug G418 at a predetermined concentration. Surface expression of B7-1, B7-2 and I-A^d proteins was analyzed by flow cytometry using appropriate mAb. Stably transfected tumor cell lines were established, and clones were generated by limiting dilution. Cloned transfected tumor cell lines were periodically monitored for the surface expression of the protein(s). The tumorigenicity of the transfected tumor cells was determined by in vivo tumor challenge experiments.

(6) BODY: Experimental Results

<u>In vivo growth kinetics</u>:

In order to determine the growth pattern of the 66.1/WT and 410.4/WT tumors naive Balb/c mice were injected subcutaneously (s.c.) in the flank with different doses of 66.1/WT and 410.4/WT tumor cells. The tumor growth was monitored by measuring the perpendicular diameters with a calipers every 5-8 days. The tumor volume was calculated by using the formula πr^3, where r=radius in mm of the tumor mass and the tumor volume was plotted as a function of time. The in vivo growth of 66.1/WT tumor cells has been previously

reported (see Annual Report, 1995). The subline 410.4/WT exhibited lesser growth compared to 66.1/WT cells before the mice became moribund (see below).

Drug sensitivity and tolerance:

As a prerequisite for the transfection experiments, the sensitivity of the 410.4/WT tumor cells to the selection drugs G418, Methotrexate and Hygromycin, was determined. The minimal concentrations of the drugs necessary to kill 410.4/WT tumor cells in vitro were determined by the same methods described for the 66.1/WT cell line (see Annual Report, 1995). The final concentrations of the selection drugs G418, Methotrexate and Hygromycin were 300 μg/ml, 2 μM and 300 μg/ml, respectively.

Constitutive expression of B7-2 on 66.1/WT tumor cells:

Previous experiments (Annual Report, 1995) showed that unlike 66.1/WT tumor cells, transfectants expressing the T cell costimulatory molecule B7-1 (66.1/B7-1 Lines) grew slowly in naive syngenic mice. Similar results were obtained with 66.1/B7-1 clones (data not shown). However, despite a significant delay, 66.1/B7-1 tumor cells grew progressively resulting in morbidity. These results indicated that 66.1/B7-1 transfectants are able to initiate an antitumor response, but it was not sufficient to mediate complete rejection. Therefore, the following additional studies were undertaken.

In addition to B7-1, another member of the B7 family, B7-2, has also been shown to provide costimulation of T cells in vitro (30-33), although B7-1 and B7-2 may have distinct roles in the differentiation of T helper cells (43, 44). Recently a few studies have compared the role of B7-1 and B7-2 in tumor rejection using different tumor models with some what different results (45-49). Therefore, it was of interest to us to investigate whether B7-2 transfected tumor cells will elicit useful anti-tumor immunity. Accordingly, 66.1/WT tumor cells were transfected with murine B7-2 cDNA and/or a plasmid containing neomycin gene to allow drug selection. The untransfected 66.1/WT tumor cells and drug resistant cells transfected with neomycin plasmid alone did not express B7-2 on their surface (Figure 1, data not shown). By contrast, 66.1 tumor cells transfected with B7-2 and neomycin cDNAs expressed B7-2. These drug resistant cells were cloned by limiting dilution. Figure 2 depicts the result of flow

cytometer analyses and shows expression of B7-2 in four representative 66.1/B7-2 clones.

Immunogenicity of 66.1/B7-2+ tumor cells:

To test the effect of constitutive expression of B7-2 on tumor rejection, groups of Balb/c mice were injected s.c. with either 66.1/WT or 66.1/B7-2 (clone 16) live tumor cells and the tumor growth was followed (Figure 3). As observed with B7-1 transfectants, there was a significant delay in the growth of 66.1/B7-2 tumor cells. One out of five mice completely rejected the tumor, and in another mouse a small tumor growth was seen. However, the majority of the mice showed delayed but progressive tumor growth. Thus, live 66.1/B7-2+ tumor cells, like 66.1/B7-1+ tumor cells, seem to initiate some antitumor immunity but again it is not strong enough to cause complete rejection.

Multiple injections of irradiated 66.1/B7-1 tumor cells induce protective immunity:

It has been observed that irradiated gene-modified immunogenic tumor cells can be used as vaccine to induce protective immunity (17, 20). In order to investigate this possibility, groups of mice were injected with either irradiated 66.1/WT or 66.1/B7-1 tumor cells. Three different radiation doses were used: 30,000, 10,000 and 3,000 rad. All mice were subsequently challenged with live 66.1/WT tumor cells. The results of the experiment is shown in Table 1. Prior injection of either 66.1/WT or 66.1/B7-1 tumor cells after higher doses of irradiation (30,000 and 10,000 rad) did not induce any protective immunity. However, multiple injections of low dose (3,000 rad) irradiated 66.1/B7-1 cells induced protective immunity against 66.1/WT challenge in about 50% of the mice. It remains to be known whether irradiated 66.1/B7-2 transfectants will also induce similar protection. Although the immunity induced by the low dose irradiated B7-1 transfectants is only moderate, further experiments can be performed to maximize this effect by increasing the number of "immunizing" injections, the dose of irradiated cells, changing the interval between the immunizing and challenge injections, etc. The use of irradiated gene-modified tumor cells as vaccine will offer a safer therapeutic strategy and with a possible clinical application to treat human tumors.

Can immunization with B7-transfected tumor cells prevent metastasis ?:

Frequently, breast cancer patients succumb to metastasis of the tumor into organs like lungs, liver, lymphnodes etc. Therefore, it was of interest to study the metastatic potential of the 66.1/WT tumor cells and compare it with the transfectants expressing B7-1 or B7-2. In a pilot study, injection of as little as 2x10e5 live 66.1/WT tumor cells resulted in morbidity in 100% of mice (n=8). The mice were sacrificed between 20 and 45 days when they became morbid and autopsy revealed metastatic nodules in lungs and liver in all the mice. Further studies are underway to study the metastatic potentials of 66.1/B7-1 or 66.1/B7-2 transfectants, and to investigate whether immunization with these transfectants will protect against metastasis of wild type tumor cells.

In vivo growth kinetics of 410.4/WT tumor cells:

Unlike the virtually non-immunogenic 66.1/WT tumor cells, the subline 410.4 is considered poorly immunogenic and does not metastasize spontaneously although an i.v. injection of 410.4 tumor cells results in metastasis into lungs, liver, etc. The following experiments have been performed to address the immunogenicity of gene-modified 410.4 tumor cells. First of all, in order to determine the in vivo growth pattern of the wild type 410.4 tumor cells (410.4/WT), groups of naive Balb/c mice were injected s.c. with describe before. Figure 4 depicts the results of this experiment. After an initial described before. Figure 3 depicts the results of this experiment. After an initial lag period of 7 - 20 days, depending on the inoculum dose, the 410.4/WT tumor cells progressively grew in the naive Balb/c mice. By day 60 - 75 most of the tumors reached the size of about 8 - 20 mm in diameter and often ulcerated, at which point the mice were sacrificed by euthanasia. In general. the 410.4/WT tumor cells exhibited lesser growth compared to 66.1/WT tumor cells.

Constitutive expression of B7-1 and B7-2 on 410.4 tumor cells:

Expression of B7-1 or B7-2 on 66.1 tumor cells, despite significant delay in the tumor growth, did not induce complete rejection of these transfectants. However, it was considered important to try a similar approach with 410.4 tumor cells for two reasons: 1) 410.4 tumor cells are considered to be slightly immunogenic, and it has been reported that the innate immunogenicity of tumor cells seem to play a role in enhancing the immunogenicity of B7 transfectants (), and 2) The relatively lesser growth of 410.4/WT tumor cells may be

advantageous to the host in that the injection of 410.4/B7 transfectants may be able to mobilize sufficient immune response ahead of the tumor growth. Therefore, 410.4 tumor cells were transfected with plasmid containing murine B7-1 or B7-2 cDNAs together with plasmid containing neomycin resistance gene. The untransfected 410.4/WT tumor cells and drug resistant cells transfected with a neomycin plasmid alone did not express B7-1 or B7-2 on their surface (Figure 1, data not shown). By contrast, 410.4 tumor cells transfected with B7-1 or B7-2 genes and neomycin cDNAs expressed B7-1 and B7-2, respectively. These drug resistant cells were cloned by limiting dilution and a number of clones expressing B7-1 or B7-2 molecules were obtained. Figures 5 and 6 depict the flow cytometer profiles of representative tumor cell clones stably expressing B7-1 or B7-2 molecules, respectively.

Immunogenicity of 410.4/B7-1+ and 410.4/B7-2+ tumor cells:

To test the effect of constitutive expression of B7-1 or B7-2 on the rejection of 410.4 tumor cells, groups of naive Balb/c mice were injected s.c. with the 410.4/B7-1 transfectants (clones 11 and 32) or 410.4/B7-2 transfectants (clones 24 and 25). The growth of these transfectants is shown in Figures 7 and 8, respectively. Unlike 410.4/WT tumor cells, both the B7-1 and B7-2 transfectants exhibited a significant delay in the onset of tumor growth, except B7-1+ clone 32 which showed a moderate delay. The size of the tumor remained very small in all the mice (B7-1+ clone 11) or in some of the mice (B7-2+ clones 24 and 25) up to day 75. In summary, these results indicated that both 410.4/B7-1 and 410.4/B7-2 transfectants were able to initiate an immune response as evidenced by a substantial delay in their in vivo growth. However, as observed with 66.1/B7-1 and 66.1/B7-2 transfectants, such an antitumor immune response was not strong enough to cause complete rejection of the B7-transfected tumor cells.

Interleukin 12 (IL-12) enhances the immunogenicity of 410.4/B7-1+ tumor cells:

The failure to induce complete rejection of B7-1 and B7-2 transfectants suggests that costimulation of CD8+ T cells by tumor encoded B7-1 or B7-2 alone is certainly not sufficient to maximally activate the tumor-specific effector T cells. The limiting factor(s) could be lack of T cell help (e.g. CD4+ T cells) (22) and/or cytokines that might amplify the T cell functions (e.g. IL-12) (50). The possible help from the activated CD4+ T cells to mediate tumor rejection is

discussed below. In vitro studies reported a synergistic effect of IL-12 (together with B7) in activating T cells (51, 52).

IL-12 is a very potent cytokine and has been used to induce rejection of a variety of tumors (53). Therefore, it is likely that administration of IL-12 would supplement the B7-mediated costimulation of T cells and result in potent antitumor immunity. Two groups of Balb/c mice were primed s.s. with B7-1 transfectants, and while one group was left untreated the other group received 9 daily injections of recombinant murine IL-12. Another group of mice were unprimed and served as controls. All mice were challenged subsequently with live 410.4/WT tumor cells i.v. Table 2 shows the results of the experiment. All the mice in the control group developed metastasis in lungs and liver and have to be sacrificed within 2-4 weeks after the i.v. challenge. Similarly, mice that were primed with B7-1 transfectants, but not treated with IL-12 developed s.c. tumor and also metastasis in lungs and liver following the i.v. challenge. By contrast, primed mice that received IL-12 did not develop metastasis in lungs and liver. In addition, 3/5 of these mice did not develop s.c. tumor, and 2/5 developed a small s.c. tumor which later regressed. These results clearly indicate that administration of IL-12 enhanced the immunogenicity of B7-1 transfectants and more importantly the immunity thus induced prevented metastasis upon subsequent challenge with wild type tumor cells.

It is very encouraging to see the combined effects of B7-1 and IL-12 resulting in enhanced tumor immunity that can not only cause rejection of local tumor growth but also has a profound effect in preventing metastasis following i.v. inoculation of fairly large numbers of wild type tumor cells. Certainly, these experiments have to be repeated with 401.4/B7-2 transfectants. Similar experiments will also be carried out with B7-1 and B7-2 transfectants of the spontaneously metastatic subline, 66.1. Additional experiments will also be carried out to see if IL-12 alone can cause the rejection of 410.1/WT and 66.1/WT tumor cells.

Constitutive expression of MHC class II:

Thus far, expression of either B7-1 or B7-2 costimulatory molecules on both the sublines, 66.1 and 410.4, only resulted in significantly delayed tumor growth compared to the corresponding wild type tumor cells. In all these situations, it is conceivable that the B7-transfectants are able to costimulate the CD8+ effector T cells. However, the failure to see complete rejection of the

transfected tumors (unless IL-12 is administered) could be due to the lack of T cell help, in the form of cytokine(s) secreted by CD4+ T cells. Therefore, it is important to activate the tumor-specific CD4+ T cells which normally recognize an antigen in the context of MHC class II molecules. Our previous study in a sarcoma tumor model showed that MHC class II+ tumor cell transfectants induced long-lasting tumor-specific tumor immunity (18, 20).

Problems:

In order to constitutively express syngenic MHC class II (I-A^d) molecules on the 66.1 and 410.4 wild type tumor cells several attempts were made. Plasmid vector containing the I-A^d alpha and I-A^d beta genes run by a SV40 promoter were obtained from several sources and tried in the transfection experiments. In all these trials, cotransfection with a separate plasmid containing a neomycin resistance gene was necessary to allow selection of the transfectants. However, after several trials, although a lot of G418 resistant colonies grew, none of the clones following limiting dilution cloning showed stable expression of I-A^d on the tumor cells surface. Some times a transient expression of I-A^d was seen in the drug resistant bulk cultures which was rapidly lost. In one attempt 3 fold higher amount of DNA was used in transfection and weakly I-A^d positive tumor cells were obtained. Different drug resistance plasmids were tried in cotransfection and selection with corresponding drugs yielded similar results.

Solution:

In an alternate attempt, the I-A^d alpha and I-A^d beta genes were cloned into a different plasmid vector containing human beta actin promoter to drive the gene. This vector also has an additional advantage of having the neomycin resistance gene in it and therefore, cotransfection with another vector containing a drug selection marker was not necessary while transfecting the wild type 410.4 and 66.1 tumor cells. In the initial experiment, the 66.1 wild type tumor cells have been transfected with this construct and Figure 9 shows the flow cytometer analysis on the expression of I-A^d molecules on the cell surface. The bulk culture of G418 resistant cells show two peaks when stained for I-A^d molecule (dotted line). The peak on the left closer to the conjugate peak (solid line) represents the negative population, while the peak on the right represents cell population expressing high amounts of I-A^d molecules. This bulk line has been cloned by limiting dilution to obtain clones expressing MHC class II (I-A^d).

When the I-A^d+ tumor cell clones become available they will be used in the in vivo tumor challenge experiments. Similarly, 410.4/A^d clones also will be generated and used in the tumor challenge experiments. In addition, transfectants that express MHC class II (I-A^d) together with B7-1 or B7-2 will also be generated and used in tumor challenge as well as therapeutic experiments.

(7) CONCLUSIONS

This report describes investigations which address the involvement of two different T cell costimulatory molecules namely B7-1 and B7-2 in eliciting antitumor immune response against a murine mammary carcinoma. In vitro studies described strong T cell costimulatory function for both B7-1 and B7-2 molecules. We and others have shown in different tumor models that constitutive expression of B7-1 or B7-2 on malignant tumor cells induced potent tumor-specific immunity. However, the present study (this report and the previous report, Annual Report 1995) show that constitutive expression of either B7-1 or B7-2 alone resulted in significant delay in the growth of mammary carcinoma cells, sublines 66.1 and 410.4. Complete rejection of B7-1 or B7-2 transfectants was not seen with either of the sublines. Although innate immunogenicity of the tumors has been implicated to contribute to the rejection of certain other B7-transfected tumors, it did not help in the case of 410.4 subline which is weakly immunogenic. Clearly additional molecules that are known to enhance T cell responses need to be tried in combination with B7-mediated costimulation.

One such molecule is the cytokine IL-12 which is known to have profound effects in T cell activation. Preliminary experiments show that administration of very small amounts of murine recombinant IL-12 not only enhanced the immunogenicity of the 410.4/B7-1 transfectants resulting in their rejection but also prevented metastasis following subsequent challenge with fairly high dose of 410.4/WT tumor cells. This observation is very encouraging and indicates the benefit of combination therapy. Therefore, this approach need to be substantiated by repeating this experiment with additional controls. Besides, similar experiments needs to be carried out with the other B7-1 and B7-2 transfectants already generated in this study. One of the major interests in these experiments will be to address metastasis of the challenge tumor.

Multiple injections of low dose irradiated 66.1/B7-1 transfectants (not similarly treated 66.1/WT tumor cells) induced protective immunity in about 50% of mice. This indicates that growth arrested B7-1 transfectants have a better chance of eliciting useful immunity. Whether a similar experiment performed with 410.4/B7-transfectants will yield the same or better immunity remains to be investigated. One advantage to this approach is that the growth arrested vaccine tumor cells might pose significantly less threat to the recipient than the live counterpart.

Constitutive expression of MHC class II molecules on the tumor cells and possible activation of tumor-specific CD4+ T cells to help generate efficient antitumor immunity is one of the major goals in this project. The initial problems have been solved and tumor cell lines stably expressing I-A^d molecule have been generated. Generation of clones expressing I-A^d alone or together with B7-1 or B7-2 are under way. The immunogenicity and therapeutic value of these transfectants will be investigated.

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Table 1

Multiple injection of irradiated B7-1 transfectants induce protective immunity

Priming (no. of cells)	Irradiation (rad)	Challenge	Tumor Incidence
None	---	66.1/WT	5/5
66.1/WT (3x10e5)	30,000	66.1/WT	5/5
66.1/WT (3x10e5)	10,000	66.1/WT	5/5
6.1/WT (5x10e5, x 4)	3,000	66.1/WT	5/5
66.1/B7-1 (3x10e5)	10,000	66.1/WT	10/10
66.1/B7-1 (5x10e5, x 4)	3,000	66.1/WT	5/10

Mice were primed s.c. with indicated numbers of irradiated (indicated dosage) wild type or B7-1 transfected tumor cells. The control group of mice did not receive any priming injection. The multiple injections groups received a total of 4 injections given with weekly intervals. Forty five days after the priming injection, or 7 days after the last priming injection in the multiple injection groups, mice were challenged s.c. with 3x10e5 live wild type (66.1/WT) tumor cells. Mice were observed for tumor incidence up to 2-3 months following challenge.

Table 2

IL-12 enhances immunogenicity of B7-1 transfectants and prevents metastasis

Priming (no. of cells)	IL-12	Challenge (no. of cells)	Mice with mets[a] /Total mice	Mice with s.c. tumor /Total mice
None	-	410.4/WT (10e6)	7/7	0/7
410.4/B7-1 (3x10e5)	-	410.4/WT (10e6)	11/11	11/11
410.4/B7-1 (3x10e5)	+	410.4/WT (10e6)	0/5	2/5[b]

Mice were unprimed or primed s.c. with 410.4/B7-1 (clone 11) tumor cells alone or mixed with IL-12 (0.25 microgram/injection). The IL-12 treatment was followed by daily i.p. injections for subsequent 8 days. Thirty days later all mice were challenged i.v. with wild type tumor cells.

a=Mice were sacrificed and examined for mets in lungs and liver when they became moribund.

b=2/5 mice developed small s.c. tumor which later regressed.

FIGURE LEGENDS

Figure 1:

Wild type tumor cells do not express B7-1, B7-2 or I-Ad molecules. 66.1/WT and 410.4/WT tumor cells were stained with either mouse anti-rat-FITC alone (panels 1, 5) or mAb against murine B7-1 (1G10, panels 2, 6), murine B7-2 (2D10, panels 3, 7) or I-Ad (M5.114, panels 4, 8) and then with mouse anti-rat-FITC. The y axes indicate the relative number of cells and the x axes indicate log flourescnce intensity. Control transfectants (plasmid containing only the neomycin gene) exhibited similar staining pattern (data not shown).

Figure 2:

Flow cytometry anlysis of 66.1/B7-2 clones. The data for four independently derived clones: 16, 11, 24 and 54 (identified as 6-2-16, 6-2-11, 6-2-24 and 6-2-54, respectively) are given. The tumor cells were stained with either mouse anti-rat-FITC alone (left panels) or stained with a rat mAb against murine B7-2 (2D10) and then with the mouse anti-rat-FITC conjugate (right panels). The y axes indicate the relative number of cells and the x axes indicate log fluorescence intensity.

Figure 3:

Tumorigenicity of 66.1/B7-2+ tumor cells. Naive Balb/c mice received 3 or 5 x 10e5 (e = exponential) live tumor cells s.c. on day 0. One group of 5 mice received wild type tumor cells (66.1/WT) and the other group received B7-2+ transfectants (66.1/B7-2 clone 16). Tumor growth was monitored as described in the text. Each line represents an individual mouse.

Figure 4:

In vivo growth kinetics of 410.4/WT tumor cells. Naive Balb/c mice received different doses (1 x 10e5, 3 x 10e5, 5 x 10e5 or 1 x 10e6) live 410.4/WT tumor cells s.c. on day 0. Each group contained 5-7 mice. Tumor growth was monitored as described in the text. Each line represents an individual mouse.

Figure 5:

Flow cytometry anlysis of 410.4/B7-1 clones. The data for four independently derived clones: 11, 32, 13 and 26 (identified as 4-1-11, 4-1-32, 4-1-13 and 4-1-26, respectively) are given. The tumor cells were stained with either mouse anti-rat-FITC alone (left panels) or stained with a rat mAb against murine B7-1 (1G10) and then with the mouse anti-rat-FITC conjugate (right panels). The y axes indicate the relative number of cells and the x axes indicate log fluorescence intensity.

Figure 6:

Naive Balb/c mice received s.c. 3 x 10e5 live 410.4/B7-1+ tumor cells (clone 11 or 32) on day 0. Each group contained 5 mice. Tumor growth was monitored as described in the text. Each line represents an individual mouse.

Figure 7:

Flow cytometry anlysis of 410.4/B7-2 clones. The data for four independently derived clones: 5, 7, 24 and 25 (identified as 4-2-5, 4-2-7, 4-2-24 and 4-2-25, respectively) are given. The tumor cells were stained with either mouse anti-rat-FITC alone (left panels) or stained with a rat mAb against murine B7-2 (2D10) and then with the mouse anti-rat-FITC conjugate (right panels). The y axes indicate the relative number of cells and the x axes indicate log fluorescence intensity.

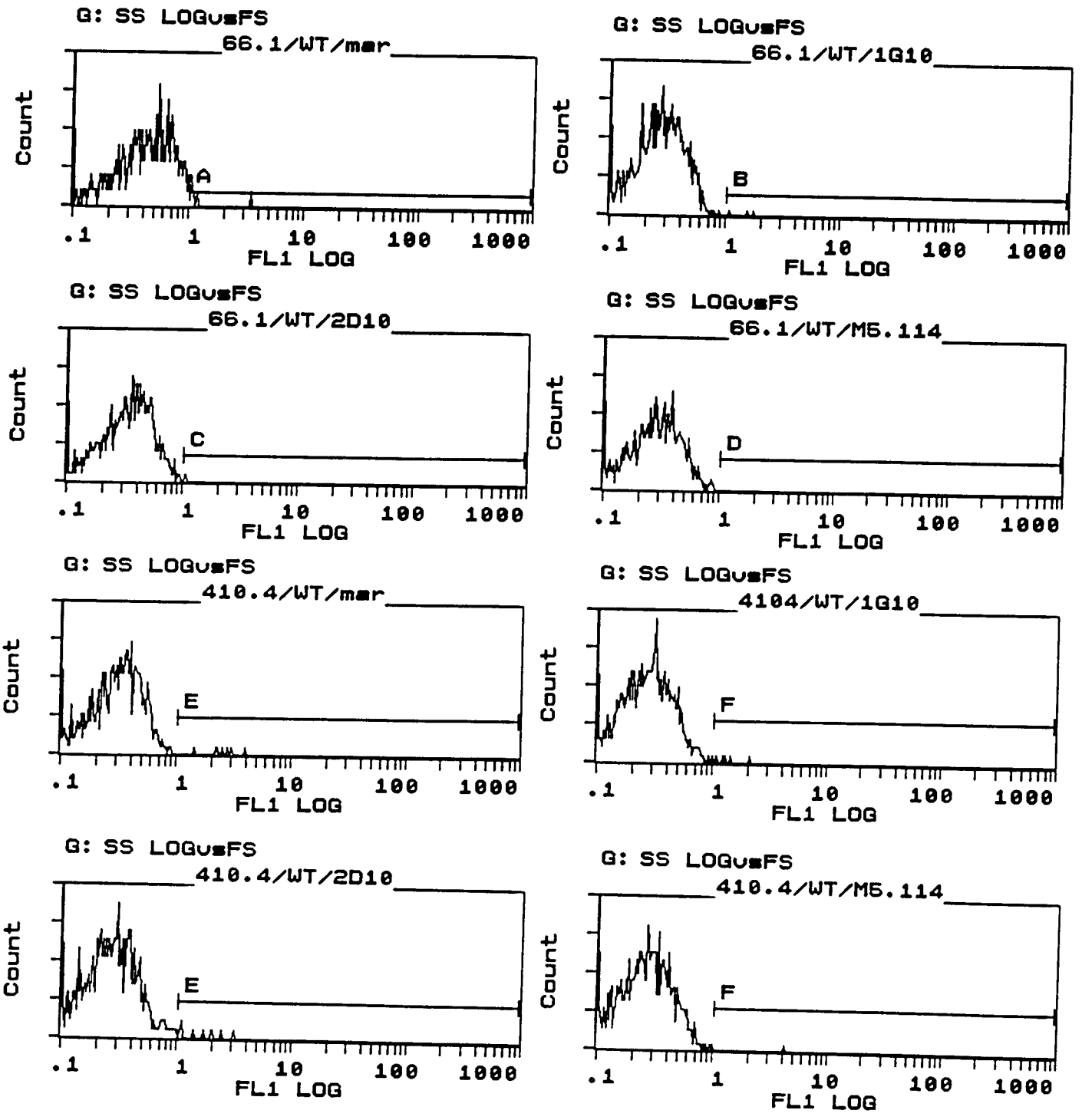
Figure 8:

Naive Balb/c mice received s.c. 3-4.5 x 10e5 live 410.4/B7-2+ tumor cells (clone 24 or 25) on day 0. Each group contained 5 mice. Tumor growth was monitored as described in the text. Each line represents an individual mouse.

Figure 9:

Flow cytometry anlysis of a bulk culture of G418 resistant cells after transfection with $A\alpha^d$ and $A\beta^d$ genes (66.1/I-A^d line). The tumor cells were stained with either mouse anti-rat-FITC alone (solid line) or stained with a rat mAb against murine I-A^d (M5.114) and then with the mouse anti-rat-FITC conjugate (dotted line). The y axis indicates the relative number of cells and the x axis indicates log fluoresence intensity.

FIGURE 1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1000
FL1 LOG
G: SS LOGvsFS
66.1/WT/1G10
Count
B
.1
1
10
100
1000
FL1 LOG
G: SS LOGvsFS
66.1/WT/2D10
Count
C
.1
1
10
100
1000
FL1 LOG
G: SS LOGvsFS
66.1/WT/M5.114
Count
D
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1
10
100
1000
FL1 LOG
G: SS LOGvsFS
410.4/WT/mar
Count
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1
10
100
1000
FL1 LOG
G: SS LOGvsFS
4104/WT/1G10
Count
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FL1 LOG
G: SS LOGvsFS
410.4/WT/2D10
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E
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1
10
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1000
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410.4/WT/M5.114
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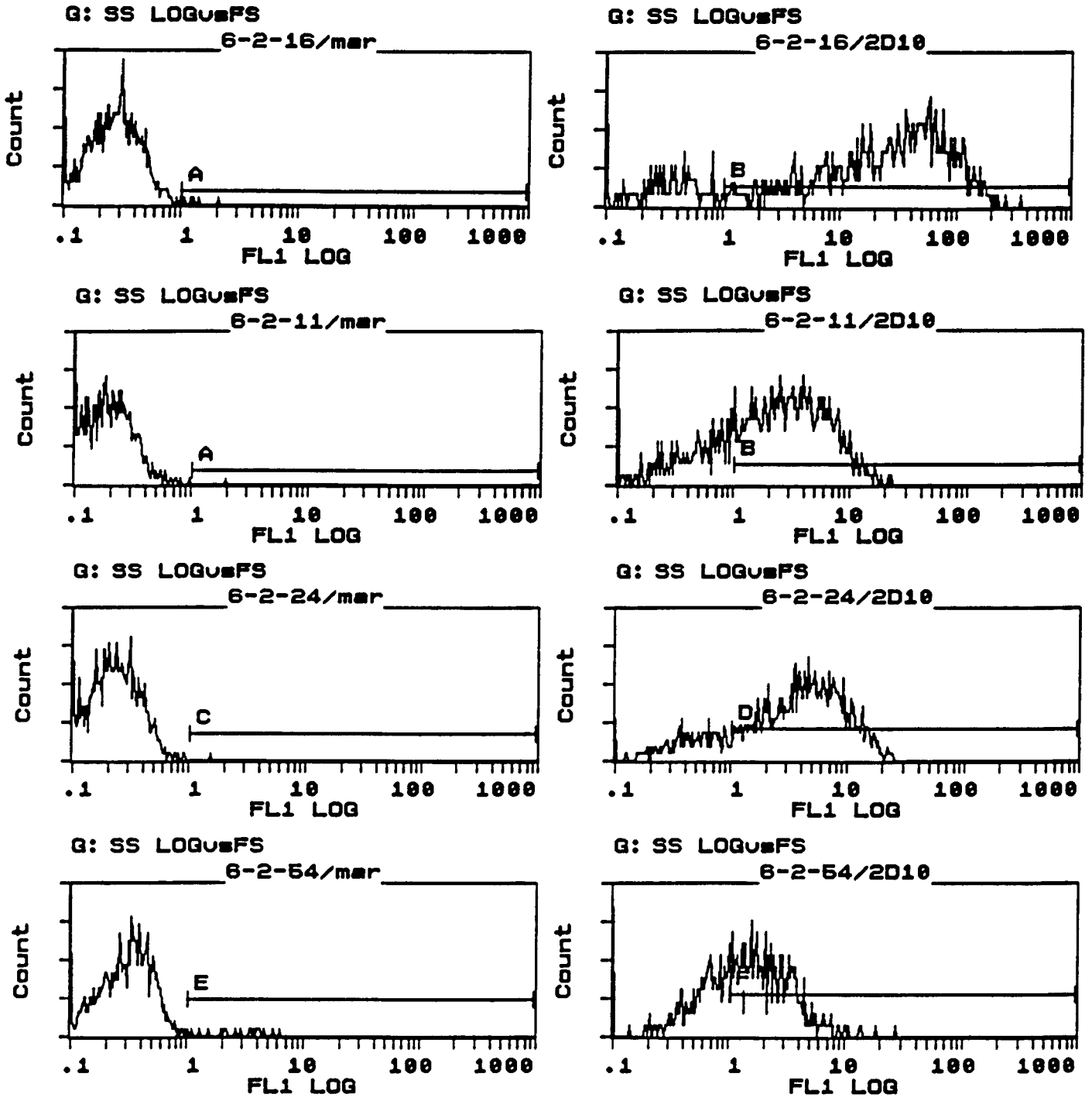
1000
FL1 LOG

FIGURE 2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1000
FL1 LOG
G: SS LOGvsFS
6-2-11/mar
Count
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10
100
1000
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Count
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G: SS LOGvsFS
6-2-24/mar
Count
C
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1000
FL1 LOG
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6-2-24/2D10
Count
D
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6-2-54/mar
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10
100
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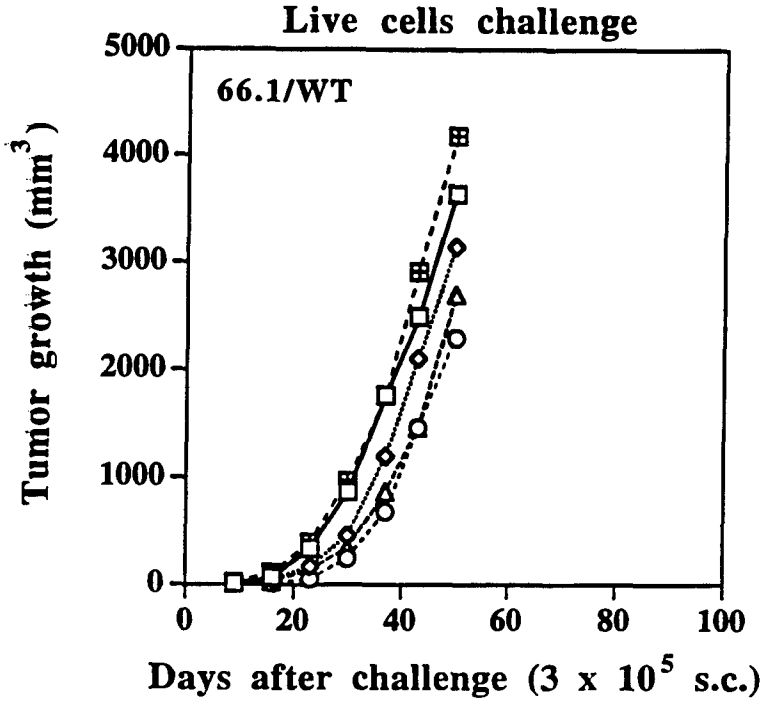
100
1000
FL1 LOG

FIGURE 3


Live cells challenge
66.1/WT
Tumor growth (mm3)
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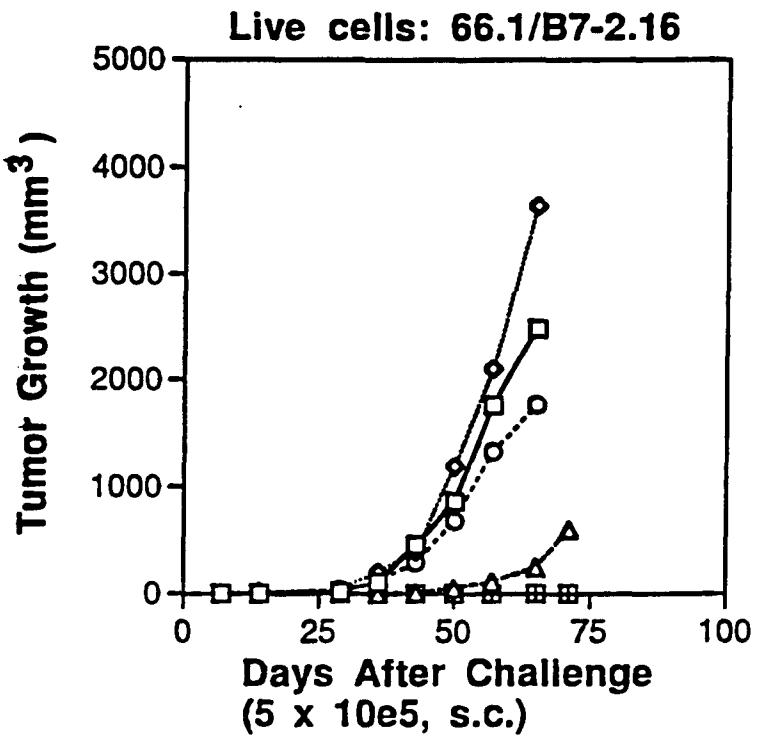
4000
3000
2000
1000
0
0
20
40
60
80
100
Days after challenge (3 x 10^5 s.c.)


Live cells: 66.1/B7-2.16
Tumor Growth (mm3)
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4000
3000
2000
1000
0
0
25
50
75
100
Days After Challenge
(5 x 10e5, s.c.)

FIGURE 4

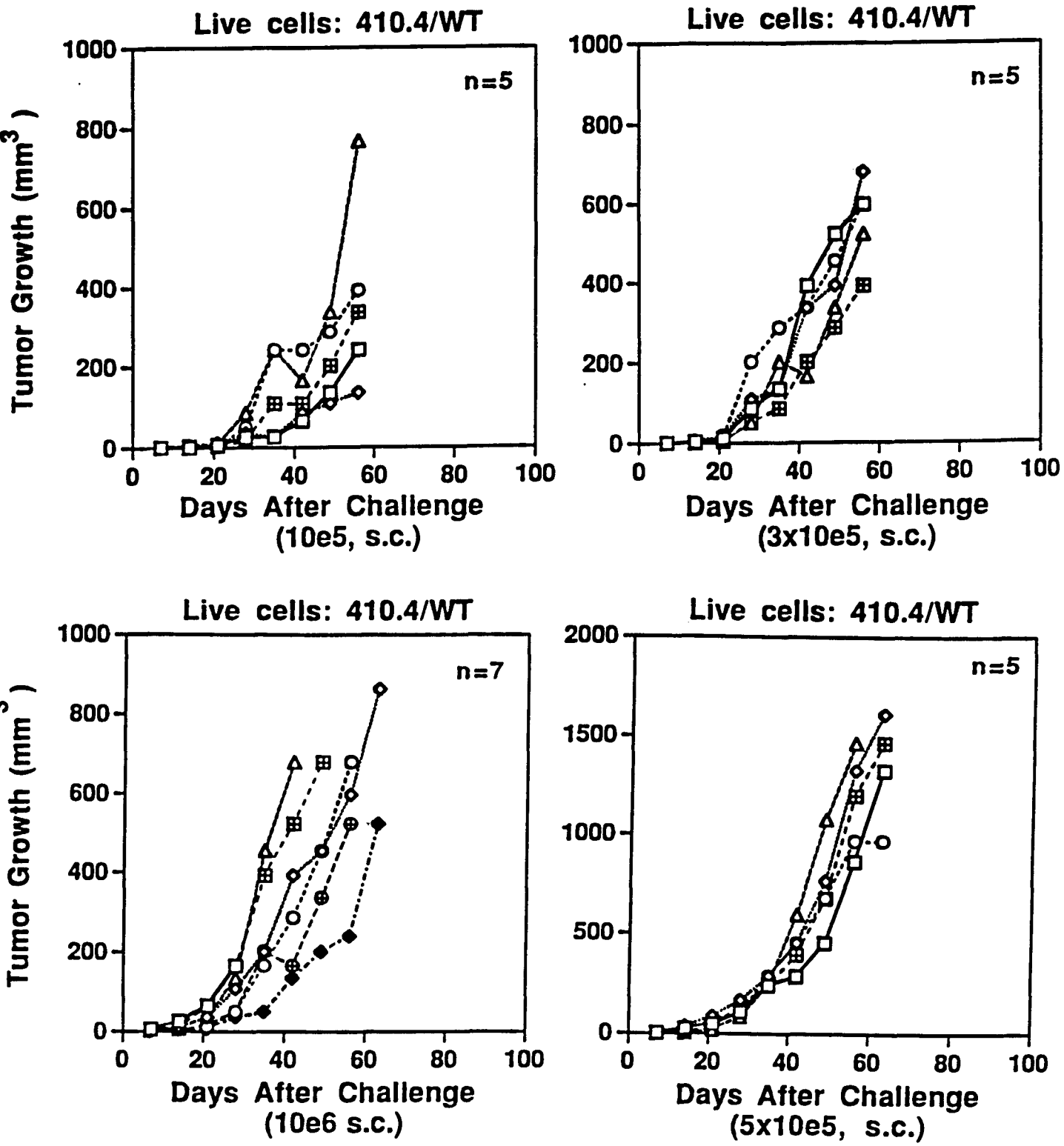
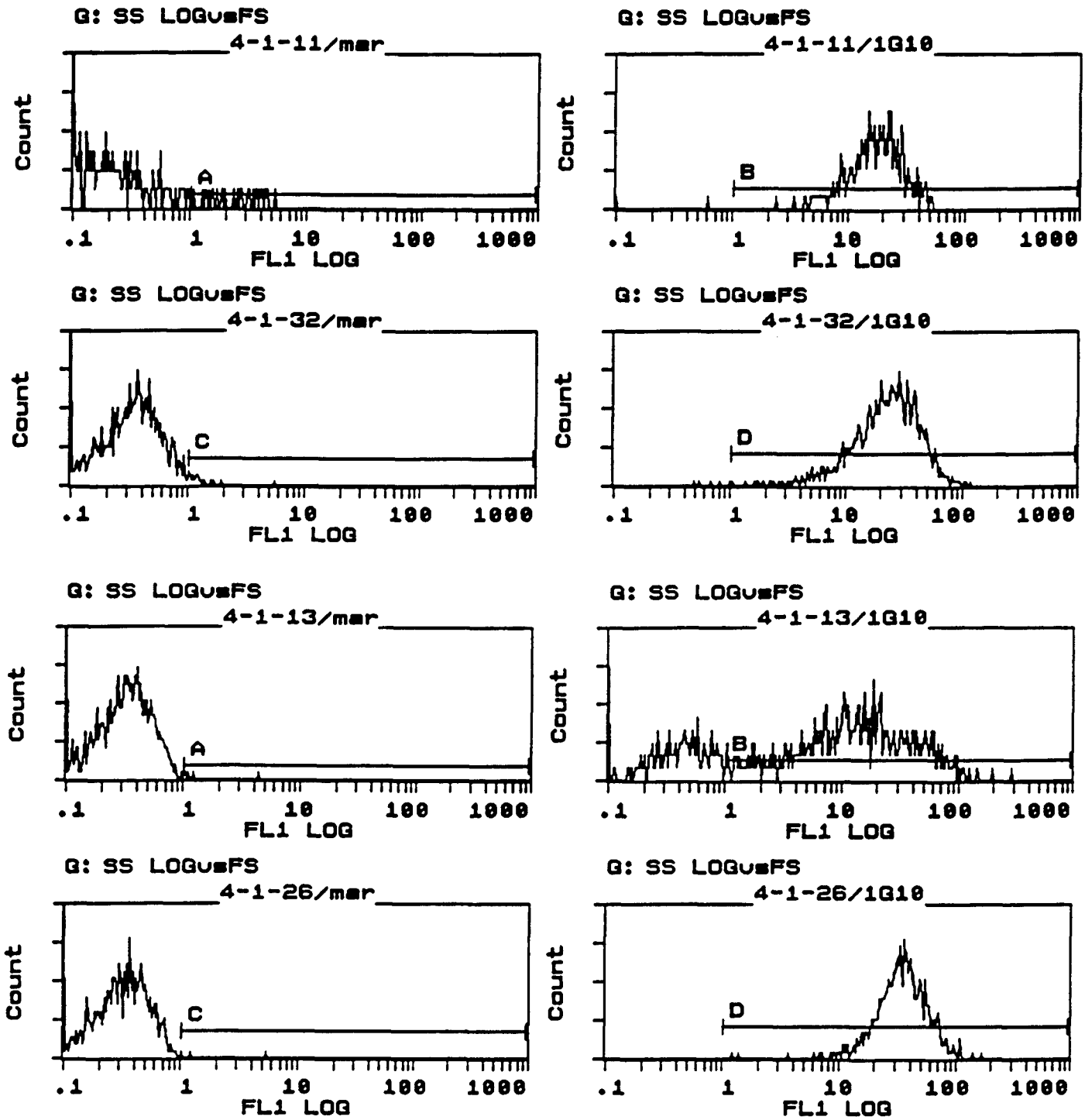

Live cells: 410.4/WT
n=5
Tumor Growth (mm3)
Days After Challenge
(10e5, s.c.)
Live cells: 410.4/WT
n=5
Days After Challenge
(3x10e5, s.c.)
Live cells: 410.4/WT
n=7
Tumor Growth (mm3)
Days After Challenge
(10e6 s.c.)
Live cells: 410.4/WT
n=5
Days After Challenge
(5x10e5, s.c.)

FIGURE 5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1000
FL1 LOG
G: SS LOGvsFS
4-1-11/1G10
Count
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1
10
100
1000
FL1 LOG
G: SS LOGvsFS
4-1-32/mar
Count
C
.1
1
10
100
1000
FL1 LOG
G: SS LOGvsFS
4-1-32/1G10
Count
D
.1
1
10
100
1000
FL1 LOG
G: SS LOGvsFS
4-1-13/mar
Count
A
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10
100
1000
FL1 LOG
G: SS LOGvsFS
4-1-13/1G10
Count
B
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1
10
100
1000
FL1 LOG
G: SS LOGvsFS
4-1-26/mar
Count
C
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1
10
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1000
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4-1-26/1G10
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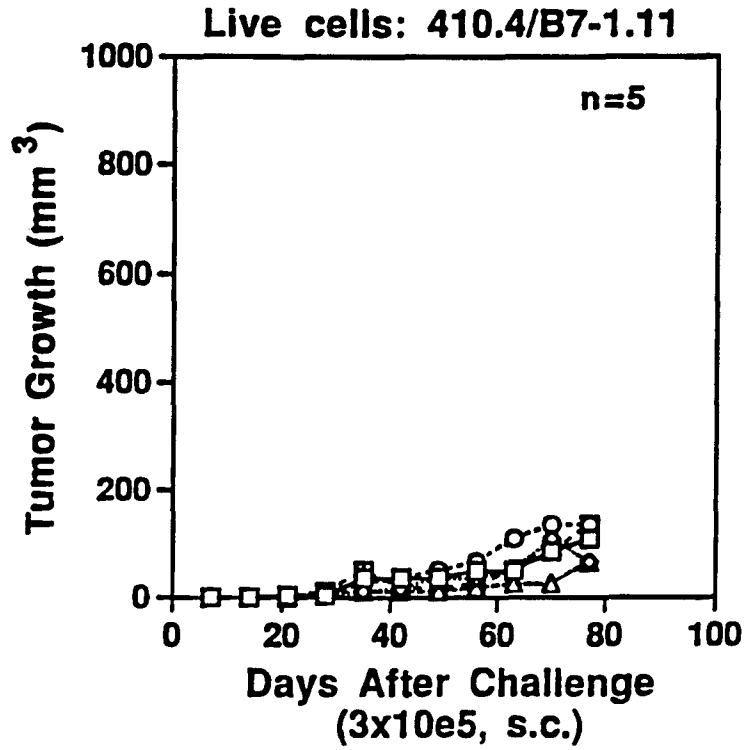
10
100
1000
FL1 LOG

FIGURE 6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n=5
Tumor Growth (mm 3)
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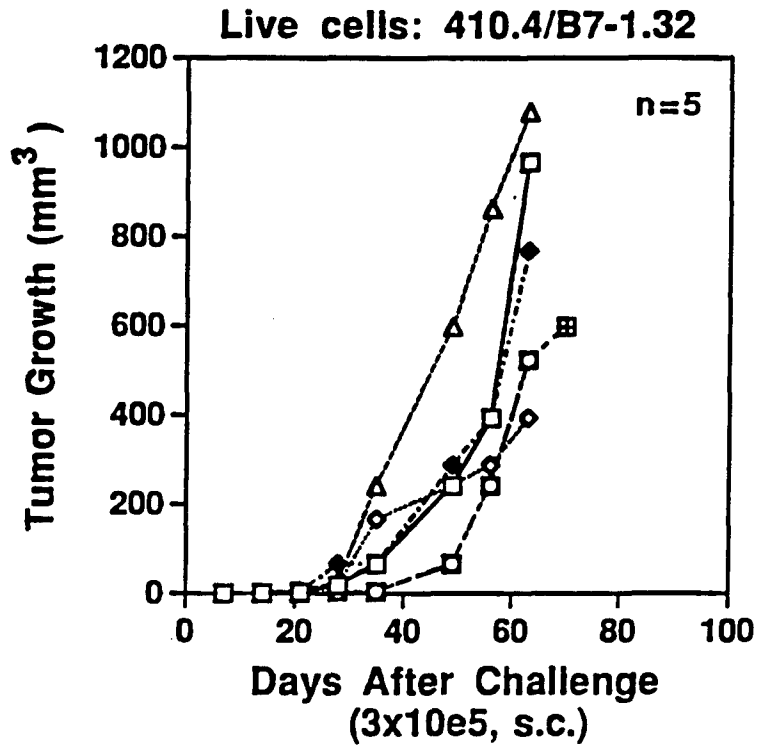
800
600
400
200
0
0
20
40
60
80
100
Days After Challenge
(3x10e5, s.c.)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Tumor Growth (mm3)
1200
1000
800
600
400
200
0
0
20
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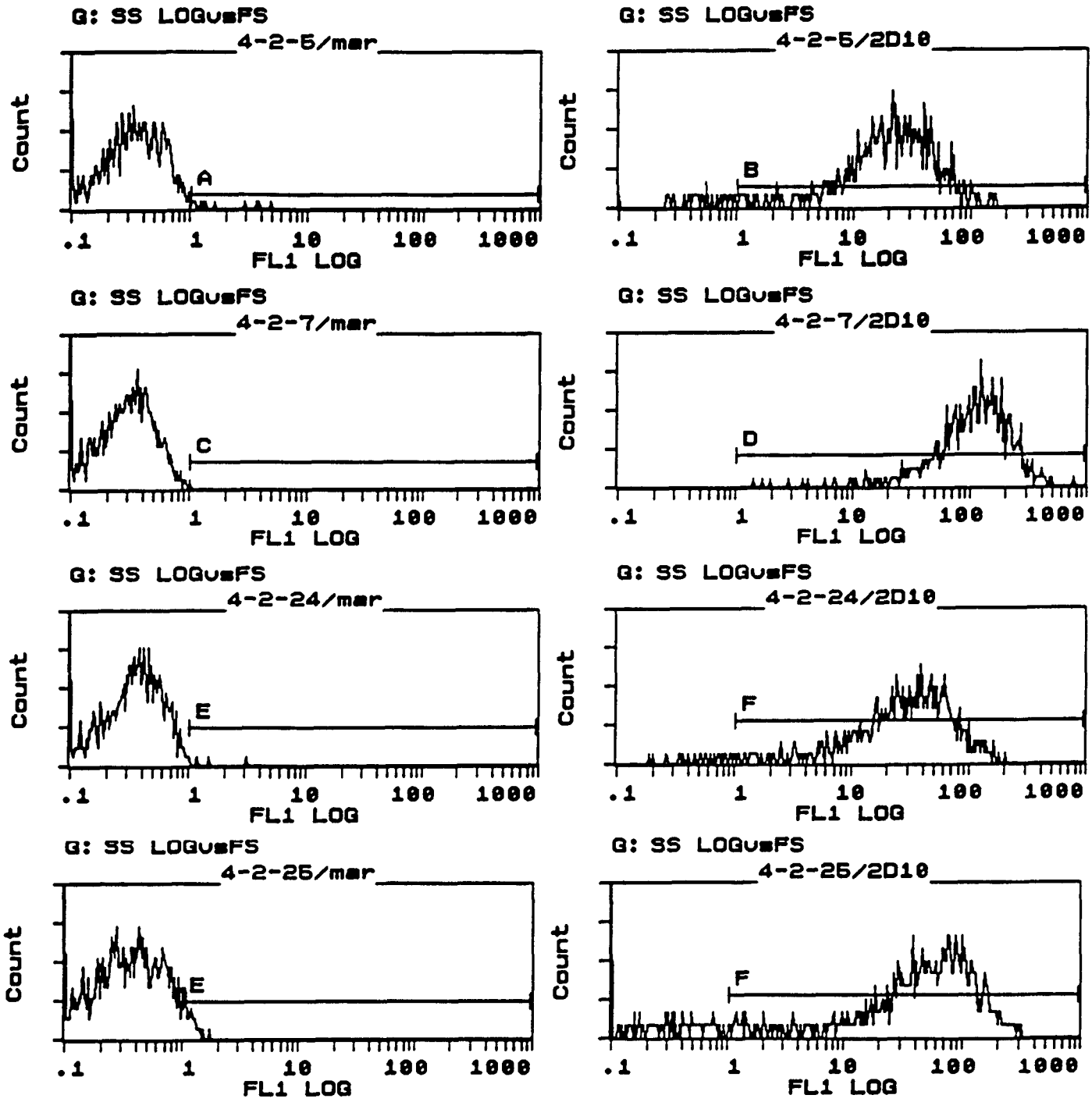
80
100
Days After Challenge
(3x10e5, s.c.)

FIGURE 7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1000
FL1 LOG
G: SS LOGvsFS
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Count
B
.1
1
10
100
1000
FL1 LOG
G: SS LOGvsFS
4-2-7/mar
Count
C
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1
10
100
1000
FL1 LOG
G: SS LOGvsFS
4-2-7/2D10
Count
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100
1000
FL1 LOG
G: SS LOGvsFS
4-2-24/mar
Count
E
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100
1000
FL1 LOG
G: SS LOGvsFS
4-2-24/2D10
Count
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100
1000
FL1 LOG
G: SS LOGvsFS
4-2-25/mar
Count
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1
10
100
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G: SS LOGvsFS
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Count
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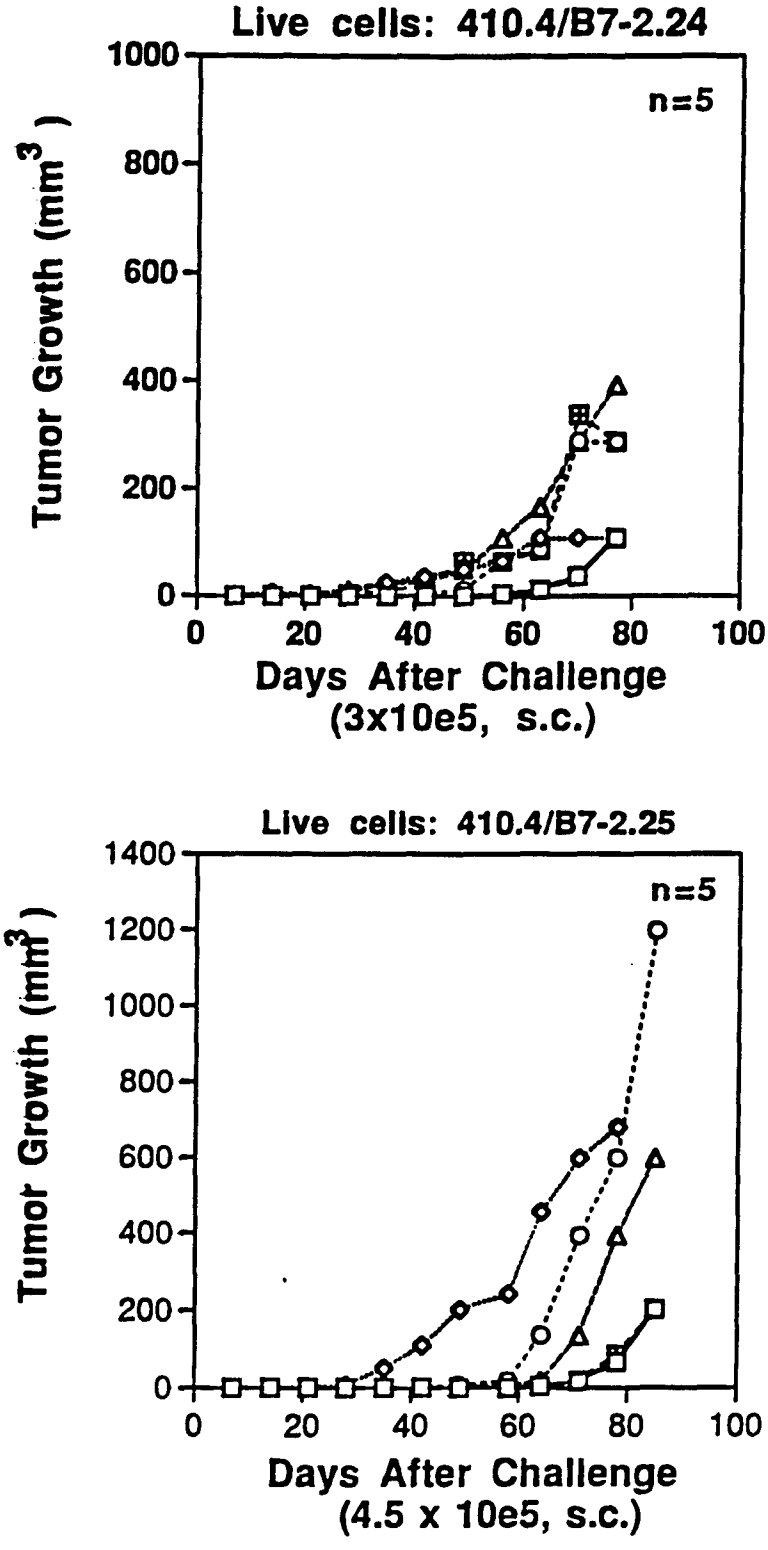
10
100
1000
FL1 LOG

FIGURE 8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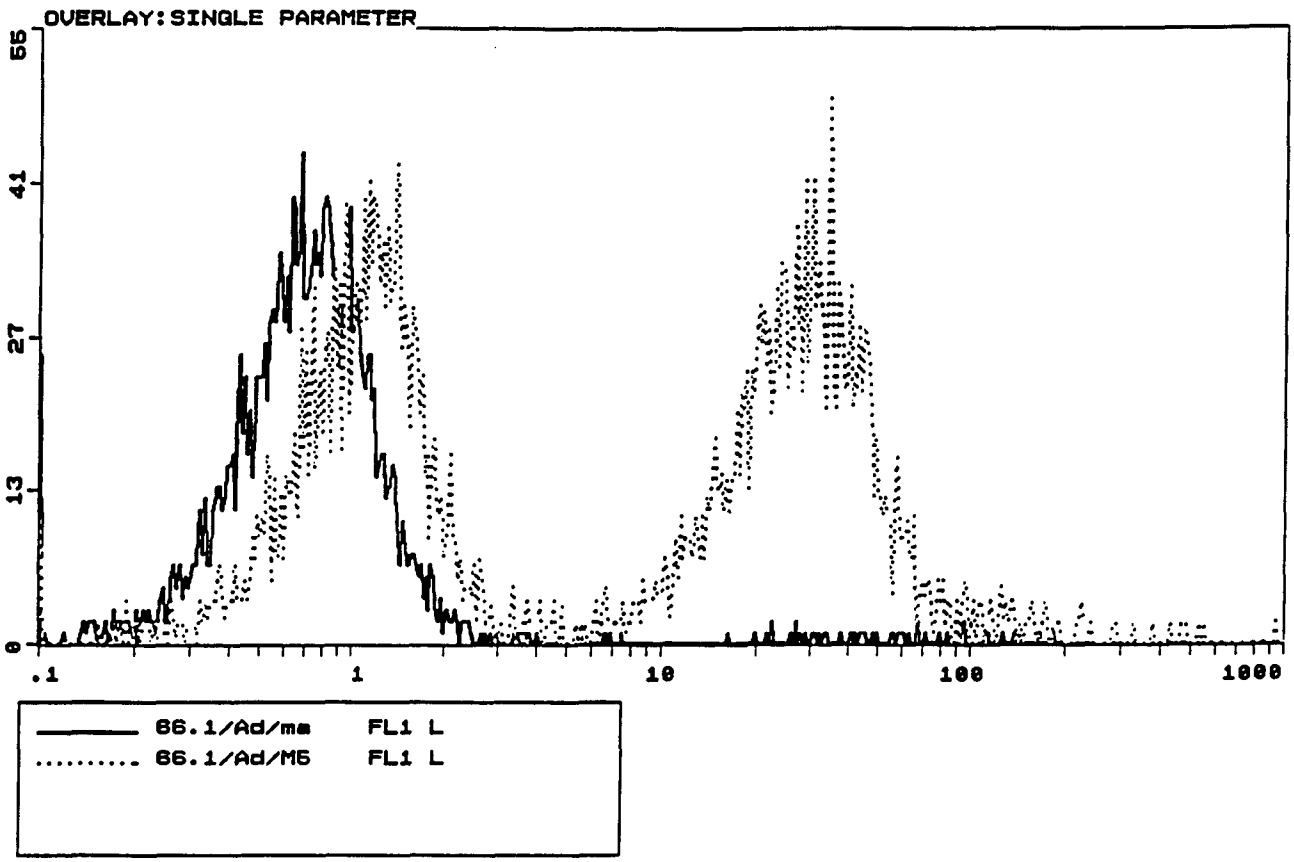
n=5
Tumor Growth (mm3)
1000
800
600
400
200
0
0
20
40
60
80
100
Days After Challenge
(3x10e5, s.c.)
Live cells: 410.4/B7-2.25
n=5
Tumor Growth (mm3)
1400
1200
1000
800
600
400
200
0
0
20
40
60
80
100
Days After Challenge
(4.5 x 10e5, s.c.)

FIGURE 9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55
41
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.1
1
10
100
1000
86.1/Ad/ma FL1 L
86.1/Ad/M5 FL1 L

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 6 May 98

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCP, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the following contracts. Request the limited distribution statement for these contracts be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

Contract Number	Accession Document Number
DAMD17-94-J-4030	ADB215484
DAMD17-94-J-4138	ADB215863
DAMD17-94-J-4158	ADB215553
DAMD17-94-J-4278	ADB215864
DAMD17-94-J-4267	ADB216187
DAMD17-94-J-4200	ADB216054
DAMD17-94-J-4185	ADB219284
DAMD17-94-J-4172	ADB224562
DAMD17-94-J-4156	ADB216186
DAMD17-94-J-4082	ADB215979
DAMD17-94-J-4053	ADB216052
DAMD17-94-J-4028	ADB218953

2. Point of contact for this request is Ms. Betty Nelson at DSN 343-7328 or email: betty_nelson@ftdetrck-ccmail.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management